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A second adenomatous polyposis coli (APC)-like gene, APC2/APCL was recently described and localized to chromosome 19. We have fine mapped APC2 to a small region of chromosome 19p13.3 containing markers D19S883 and WI-19632, a region commonly lost in a variety of cancers, particularly ovarian cancer. Interphase FISH analysis revealed an APC2 allelic imbalance in 19 of 20 ovarian cancers screened and indicates that APC2 could be a potential tumor suppressor gene in ovarian cancer. When over-expressed in SKOV3 ovarian cancer cells, which express low levels of APC2, exogenous APC2 localized to the Golgi apparatus, actin-containing structures, and occasionally to microtubules. Antibodies against the N-terminus of human APC2 show that endogenous APC2 is diffusely distributed in the cytoplasm and co-localizes with both the Golgi apparatus and actin filaments. APC2 remained associated with actin filaments following treatment with the actin-disrupting agent, cytochalasin D. These results suggest that APC2 is involved in actin associated events and could influence cell motility or adhesion through interaction with actin filaments as well as functioning independently or in cooperation with APC to down-regulate β-catenin signaling.

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Christy Jarrett 8/30/01

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Table of Contents

Cover.....1

SF 298..... 2

Foreword..... 3

Table of Contents..... 4

Introduction..... 5

Body..... 6

Key Research Accomplishments..... 13

Reportable Outcomes..... 14

Conclusions..... 15

Appendices..... 17

Introduction

Our lab identified an N-terminal sequence with significant homology to the adenomatous polyposis coli (APC) tumor supressor gene, which is commonly associated with colon cancer. Possible functions of APC include the regulation of β-catenin protein degradation and signaling and microtubule mediated cell migration. β-catenin binds to the Tcf/LEF transcription factor complex and regulates the transcription of c-myc and cyclin D1, thus indicating that this pathway may be involved in cell cycle regulation. Truncating mutations in APC or mutations in certain N-terminal serine residues of β-catenin, result in increased β-catenin levels and increased transcriptional activation. Increased β-catenin levels have been found in multiple cancers, including breast and ovarian cancers, which have normal APC function. This suggests that there is another potential tumor supressor gene involved in β-catenin regulation. During the course of the work reported here, the full-length sequence of a new APC-like gene, APCL/APC2 (originally called ALG19) was published. This project focuses on characterizing and determining the function of this new APC2 gene.

Body

Using a 1 kb sequence to the N-terminal region, four PAC clones (1K8, 17J21, 22K8, and 26K20) were isolated for APC2. FISH analysis using the four clones localized APC2 to chromosome 19p13.3. 19p13.3 is ~20 mb in size. The genomic sequence of APC2 is ~ 40 kb and the coding sequence 7 kb. APC2 was then fine mapped by radiation hybrid mapping to the 800 kb region containing markers D19S883 and WI-19632 using primers designed to span the first exon/intron junction. This particular region of 19p13.3 exhibits significant loss of heterozygosity (LOH) in many different cancers and is near the Peutz-Jeghers syndrome (PJS) associated gene, LKB1/STK11. PJS is characterized by intestinal hamartomas and increased risk of gastrointestinal, ovarian, pancreatic, and breast cancers. Even though there is significant LOH in this region, there are few mutations in the LKB1 gene in sporadic breast and colorectal cancers and adenoma malignum of PJS patients. In addition, although 50% of ovarian cancers contain an LOH on 19p13.3, LKB1 is not mutated indicating that another gene of significance in the development of cancer exists in this region. For example, marker D19S216, which is 9.5 fcM distal to marker D19S883, but not LKB1 itself, exhibits 100% LOH in sporadic adenoma malignum of the uterine cervix. Therefore, APC2 could be a tumor suppressor gene affected by LOH of chromosome 19p13.3.

Using PAC clone 22K8, we performed FISH analysis on the SKOV3 ovarian cancer cell line, which had reduced levels of APC2 protein, and a variety of other cancer cell lines including those derived from breast and colon. These pilot studies showed that most had two or more copies on chromosome 19. However, although SKOV3 cells had on average 3 signals, none of them were on chromosome 19. To continue this study, we screened 20 sporadic breast and ovarian tumors by interphase FISH analysis. Remarkably, 19 of 20 specimens from ovarian cancer patients exhibited

marked allelic imbalance. In contrast no significant allelic imbalance was observed in normal ovarian or breast tissue. The number of signals of the 19p telomeric marker was also significantly less than 2 in the ovarian cancer specimens. Even taking into account the bias toward slightly underestimating hybridization signals using interphase FISH, these data are consistent with published studies showing a marked loss of chromosome 19ptel in ovarian cancers and indicate an even more marked loss of APC2. Although the overall ratio of APC2/19p when averaged over 20 breast cancers was not markedly reduced, 4 of 20 breast cancer specimens did actually exhibit allelic imbalance at this locus. It is also important to note that these 4 tumors were the most aggressive and poorly differentiated of the 20 tumors examined.

In pilot studies we found that many cells expressed significant levels of APC2. However, the ovarian cancer cell line SKOV3 expressed low levels of APC2 as demonstrated by western blot and immunocytochemistry. We used SKOV3 cells to examine the distribution of exogenously expressed APC2. Upon APC2 transfection, the intensity of several bands was markedly increased as detected by western blot using a chicken antibody to APC2. Immunocytochemistry showed that APC2 transfected cells could easily be detected against the background of non-transfected cells. Exogenously expressed APC2 localized around the nucleus and co-localized with PKCμ, a kinase known to associate with the Golgi apparatus. Co-localization of APC2 and PKCμ was observed for much of the Golgi stack indicating that APC2 is associated with certain regions of the Golgi where it co-localizes with PKCμ even though the Golgi apparatus is somewhat disrupted in the transfected cells. APC2 co-localized with actin filaments at the cell membrane. β-catenin co-localized with exogenous APC2 in the aggregates at the Golgi apparatus whereas in untransfected cells β-catenin is localized only at the membrane. This could be indicative of a transport function for APC2 as suggested in recent studies. Exogenously expressed APC2 also appears to co-localize with some perinuclear microtubules.

APC2 expression was determined by RT-PCR, Northern analysis and Western analysis of both cell lines and tissue. For RT-PCR 1 ug of total RNA from each cell line or tissue was used for amplification, for Northern analysis 10 ug of total RNA and for Western analysis 60 ug of protein from each cell line. We confirmed that APC2 is expressed in a variety of tissues, including breast, colon, brain, and ovary, at both the RNA and protein level. In some cells APC2 levels were reduced but still detectable. APC2 expression, like APC, is greatest in the brain; however, there are differing levels in different brain regions with very little expression in the cerebellum and cerebral cortex. Lymphoid tissues and lymphoma cell lines had no detectable APC2 at the mRNA or protein level with the exception of K-562 leukemia cells, which express low levels of APC2.

Both rabbit and chicken hAPC2 antibodies were affinity purified on an antigen coupled CNBr column. Western blot analysis determined that both antibodies were specific to hAPC2 with no cross-reactivity to APC. This was confirmed using the SW480 colon cancer cell line that contains a C-terminally truncated form of APC. Neither rabbit nor chicken hAPC2 antibodies detect this truncated APC protein even though it contains the conserved N-terminus. The predicted molecular weight of full-length APC is ~310 K and that of APC2 is ~245 K. In addition, APC2 is present in T84 cells, which have a homozygous deletion of the APC gene. To further determine specificity, we blocked the antibody with recombinant antigen before Western blot analysis and found that all bands are specific to APC2. Similarly, when preimmune IgY was used to probe Western blots no staining was observed. Western blot analysis showed that APC2 is expressed in many cells lines including SKBR3, SW480, MDCK, MDA-MB-157 and 436. HL-60 lymphoma cells have no detectable APC2 protein, which correlates with the mRNA data. A characteristic pattern of immunoreactive species was observed. Three major bands larger than 200 kD and several smaller molecular weight species of ~121, 81, and 51 kD were present consistently. Other cell lines, for example MDA-MB-157 and SKOV3, have

significantly less of all bands. The presence of multiple bands by western blotting with N-terminal APC2 antibodies is similar to that observed for APC using N-terminal APC antibodies. In the case of APC the multiple banding pattern has been ascribed to a combination of multiple splice variants and to degradation products. Because several lower molecular weight species are present in cells that are transfected with full-length APC2 it is likely that these bands represent degradation products. Some differences in the pattern of immunoreactive species were observed when endogenous APC2 was compared in different cell lines and when compared to exogenously expressed APC2. These variations are likely to represent variable degrees of proteolytic degradation in the different situations. However it is possible that like APC, APC2 may also have many splice variants.

The N-terminal region of APC2 contains the highly conserved dimerization domain. This region in APC has been shown to dimerize *in vitro*. To determine whether APC associates with APC2, APC2 was immunoprecipitated with the purified rabbit antibody and APC detected using APC Ab-1 in SW480 and HBL-100 cells. Full-length APC was detected in HBL-100 cells and the 150 kD truncated form of APC was detected in SW480 cells. This result demonstrates that APC and APC2 can either dimerize or associate in a complex in a detergent soluble lysate.

To investigate the localization of endogenous APC2 in the cell, we performed immunocytochemistry on several cell lines including SKBR3 breast cancer cells, MDCK normal canine kidney cells, SW480 colon cancer cells, and A549 lung carcinoma cells. Although both rabbit and chicken antibodies exhibited a similar staining pattern by immunocytochemistry, the chicken antibody was exceptional and was used for these studies. Preimmune chicken IgY and antigen blocked antibody, as well as IgY prior to antigen affinity purification, were completely negative. Specific APC2 staining was similar in all cell lines and was observed diffusely in the cytoplasm as well as being associated with tubular structures adjacent to the nucleus that resembled the Golgi apparatus.

Staining was also concentrated along filamentous structures and in what appeared to be lamellipodial membranes.

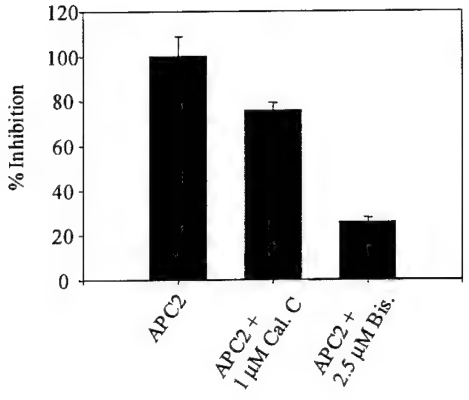
As was observed with exogenously expressed APC2 in SKOV3 cells, endogenous APC2 co-localized with PKCμ at the Golgi apparatus. A relationship between APC2 and actin filaments was observed in cells stained with phalloidin. However, not all actin filaments stained and APC2 appeared to be concentrated near actin-associated membrane ruffles and lamellipodia as well as cell-cell contact sites. Treatment with cytochalasin D, an actin-disrupting agent, causes actin filaments to collapse and coalesce mostly at the cell periphery but also throughout the cell. Following treatment with cytochalasin D, APC2 remained associated with the actin filaments in MDA-MB-157 cells.

Endogenous APC is localized at the tips of microtubules in MDCK cells and is not associated with actin filaments. However, overexpression of APC results in the decoration of microtubules throughout the cell. Consistent with this, cytochalasin D treatment does not affect APC staining but disruption of microtubules with nocodazole does.

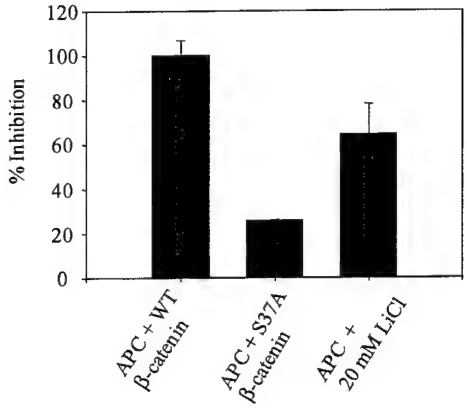
Finally, we looked at the ability of APC2 to regulate β-catenin signaling. As shown previously, APC2 can inhibit β-catenin signaling in SW480 cells. Several studies have pointed to a role of PKC-like enzymes in the transmission of the wingless signal. We investigated the effects of Calphostin C, a diacylglycerol (DAG)-dependent protein kinase C (PKC) inhibitor, and bisindoylmaleimide (bis), which inhibits both DAG-dependent and independent PKC isoforms, on the ability of APC2 to inhibit β-catenin signaling. Calphostin C had little effect on APC2 inhibition of β-catenin signaling (Figure 1A). This is consistent with our earlier work in which we showed that this inhibitor did not increase cytoplasmic β-catenin. In contrast, bis almost completely reversed the APC2 mediated inhibition of β-catenin signaling. Bis also inhibits APC activity and increases cytoplasmic β-catenin. These results point to a role for atypical PKC-activity in the regulation of both APC and APC2 function.

GSK3β forms a complex with axin, β-catenin, and APC, which can then regulate β-catenin turnover. Although the precise role of GSK3β is not clear, Li^+, which inhibits GSK3β activity, leads to the accumulation of β-catenin in the cytoplasm. We recently showed that LiCl does not significantly inhibit the ability of APC to down-regulate β-catenin signaling. In the present study we found APC2 to be somewhat more sensitive to LiCl than APC. LiCl increases signaling 3 fold over APC2 alone compared to ~1.5 fold for APC (Figure 1B and C). Another significant difference between APC and APC2 is the ability of APC2 to inhibit the signaling activity of a mutant S37A form of β-catenin that is resistant to inhibition by APC.

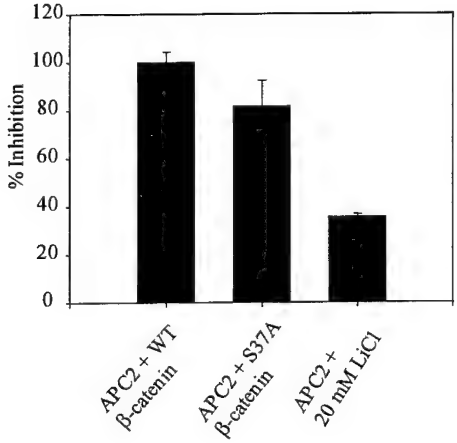
A


120
100
80
60
40
20
0
% Inhibition
APC2
APC2 + 1 µM Cal. C
APC2 + 2.5 µM Bis.

B


120
100
80
60
40
20
0
% Inhibition
APC + WT β-catenin
APC + S37A β-catenin
APC + 20 mM LiCl

C


120
100
80
60
40
20
0
% Inhibition
APC2 + WT β-catenin
APC2 + S37A β-catenin
APC2 + 20 mM LiCl

Key Research Accomplishments

- Identification of a new APC-like gene, APC2 (~245 K), which is overall 35% identical to APC (~310K).
- APC2 is located on chromosome 19p13.3 in a region containing markers D19S883 and WI-19632, a region of LOH predisposing to several different cancers including breast and ovarian.
- APC2 is expressed in most cells and tissues, including breast, colon, and ovary with greatest expression in the brain, which is similar to APC.
- APC2 is localized diffusely in the cytoplasm, is localized to the golgi apparatus, and is associated with actin filaments.
- Upon retinoic acid (RA) treatment, APC2 co-localizes with β-catenin and actin filaments at the membrane in SKBR3 breast cancer cells.
- APC2 regulates β-catenin signaling activity similar to APC.
- APC2 and APC associate in a detergent soluble lysate.
- Upon overexpression, APC2 localizes at the Golgi apparatus, in aggregates containing β-catenin, and associates with both actin and microtubule filaments.
- There is a significant APC2 allelic imbalance in ovarian cancer.
- An APC2 allelic imbalance was found in 4 of 20 breast tumors, however these 4 were the most aggressive of the 20 specimens examined.

Reportable Outcomes

- Manuscript accepted for publication in Cancer Research: "Human APC2 localization and allelic imbalance" (see Appendix)
- Christy Jarrett, the principal investigator, obtained PhD in May 2001.
- Christy Jarrett awarded a post-doctoral fellowship at Harvard University Department of Medicine and Beth Israel Deaconness Medical Center Department of Pathology.

Conclusions

Endogenous APC localizes near the ends of microtubules in a punctate pattern but does not co-localize with actin, however, when over-expressed, APC associates with microtubules throughout the cell. Over-expressed APC2 forms aggregates at the Golgi apparatus and co-localizes with actin filaments as well as some microtubules. Our results indicate that as is the case with APC, APC2 localization with microtubules in the cell body may only occur when APC2 is present at very high levels. It has been suggested that APC might be involved in microtubule regulated membrane protrusion and cell migration as well as inhibition of β-catenin signaling. Like APC, APC2 also can inhibit β-catenin signaling. Although APC2 and APC do not co-localize at the membrane or cytoskeletal structures they are both present in the cytoplasm. Immunoprecipitation results show that they can exist in the same complex in this environment. Taken together these findings suggest an intriguing scenario in which cytoplasmic APC and APC2 regulate related microtubule and actin-based functions and β-catenin signaling either independently or in cooperation. APC and APC2 could cooperate in the cytoplasm or in association with microtubules and actin filaments respectively to control such processes as β-catenin signaling, cellular transport, and cell motility.

The chromosomal localization of APC2 to chromosome 19p13.3 within 12 fcM of the telomere is significant because this region is associated with Peutz-Jeghers Syndrome (PJS) and exhibits significant loss of heterozygosity (LOH) in several sporadic cancers. Loss of 19p13.3 occurs in many sporadic cancers including those of the breast and is remarkably common in sporadic ovarian carcinomas (~50%). Our fine-mapping analysis shows that APC2 is located in the region of markers D19S883 and WI-19632 between the LKB1 gene and the site of 100% LOH found in adenoma malignum of the uterine cervix. Importantly, we found significant allelic imbalance of APC2 in

sporadic ovarian cancers. We also found allelic imbalance of APC2 in 4 of 20 sporadic breast cancers. These 4 tumors were the most aggressive of the 20 tumors examined; however, more studies are needed to determine if this correlation is significant. Therefore, like APC, APC2 could be a tumor suppressor gene important in several cancers particularly ovarian cancers. At this time some single nucleotide polymorphisms (SNPs) have been discovered in several genes on chromosome 19p13.3 but none in the APC2 gene itself. Further studies searching for SNPs and a more formal LOH study will better determine whether APC2 is a tumor suppressor gene.

Human APC2 localization and allelic imbalance [1]

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Key words: APC, APC2, β-catenin, ovarian cancer, chromosome 19p13.3

Footnotes

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[3]Abbreviations: APC, adenomatous polyposis coli; APC2, APC-like gene; dAPC, Drosophila APC; dAPC2/E-APC, Drosophila APC2; FISH, fluorescence in situ hybridization; hAPC2, human APC2; HGS/TIGR, Human Genome Sciences and The Institute for Genomic Research; PAC, P-1 derived artificial chromosome; PJS, Peutz-Jeghers syndrome; SNP, single nucleotide polymorphisms

ABSTRACT

A second adenomatous polyposis coli (APC)-like gene, APC2/APCL was recently described and localized to chromosome 19. We have fine mapped APC2 to a small region of chromosome 19p13.3 containing markers D19S883 and WI-19632, a region commonly lost in a variety of cancers, particularly ovarian cancer. Interphase FISH analysis revealed an APC2 allelic imbalance in 19 of 20 ovarian cancers screened and indicates that APC2 could be a potential tumor suppressor gene in ovarian cancer. When over-expressed in SKOV3 ovarian cancer cells, which express low levels of APC2, exogenous APC2 localized to the Golgi apparatus, actin-containing structures, and occasionally to microtubules. Antibodies against the N-terminus of human APC2 show that endogenous APC2 is diffusely distributed in the cytoplasm and co-localizes with both the Golgi apparatus and actin filaments. APC2 remained associated with actin filaments following treatment with the actin-disrupting agent, cytochalasin D. These results suggest that APC2 is involved in actin associated events and could influence cell motility or adhesion through interaction with actin filaments as well as functioning independently or in cooperation with APC to down-regulate β-catenin signaling.

INTRODUCTION

The APC[3] tumor suppressor gene, located on chromosome 5q21, is associated with colon cancer. Possible functions include the regulation of β-catenin protein degradation and signaling and microtubule mediated cell migration (1-3). β-catenin binds to the Tcf/LEF transcription factor complex and regulates the transcription of c-myc and cyclin D1, thus indicating that this pathway may be involved in cell cycle regulation (4-8). Truncating mutations in APC or mutations in certain N-terminal serine residues of β-catenin, result in increased β-catenin levels and increased transcriptional activation (2,3,9,10).

APCL/APC2, an APC homologue, located on chromosome 19p13.3 has also been shown to interact with β-catenin and can decrease β-catenin levels and signaling activity in SW480 colon cancer cells (11,12). hAPC2 is expressed in many different tissues and cell lines including brain, breast, colon, and ovary. APC family members have similar N-terminal dimerization domains, armadillo repeats, and β-catenin binding and regulatory domains but are less conserved at the C-terminus, which contains the basic domain, microtubule binding domain, and disc large binding site in hAPC. In addition, hAPC2 lacks the three 15 amino acid β-catenin binding sites. A phylogenetic tree of the highly conserved armadillo repeat domain suggests independent drosophila and vertebrate APC gene duplications (13). Drosophila APC (dAPC) is known to regulate wingless signaling in the eye but has not been associated with other tissues (14). Drosophila APC2 (dAPC2)/E-APC negatively regulates wingless signaling in the epidermis (13,15). These

results suggest that APC and APC2 could be tissue specific and/or have different roles in β-catenin regulation.

Endogenous APC localizes near the ends of microtubules but does not associate with actin filaments or with microtubules in the cell body, however, when overexpressed, APC associates with microtubules throughout the cell (1,16,17). Over-expressed hAPC2 localizes to the perinuclear region and to some microtubules in the cell body but no studies have examined the distribution of endogenous hAPC2 (18).

dAPC2/E-APC co-localizes with actin caps during development and weak E-APC mutations in Drosophila females cause a loss of E-APC at the membrane of nurse-cells in the ovary, which corresponds to a loss of Armadillo/β-catenin at the membrane (19,20). β-catenin localizes at the membrane to form adhesion junctions, which suggests that cell adhesion of these cells is compromised by the mutation in E-APC. In this study we show that endogenous hAPC2 co-localizes with the Golgi apparatus and actin filaments, particularly those filaments present at the leading edge of the cell and at cell-cell contact sites but was not associated with microtubules unless over-expressed. Significantly, SKOV3 ovarian cancer cells express considerably less APC2 than other cell lines. We have fine-mapped APC2 to a region of chromosome 19p13.3 containing markers D19S883 and WI-19632, a region commonly lost in a variety of cancers particularly ovarian cancer (21-23). Using interphase fluorescence in situ hybridization (FISH), we show an allelic imbalance of APC2 in 19 of 20 sporadic ovarian tumors indicating that APC2 could be a potential tumor suppressor gene.

MATERIALS AND METHODS

FISH Analysis and Fine Mapping: A 1kb cDNA fragment from the N-terminal region of APC2 corresponding to the recombinant protein used to make antibodies was used to screen a P-1 derived artificial chromosome (PAC) library (Human Genome FISH Mapping Resource Centre at the Ontario Cancer Institute). Four genomic PAC clones were identified: 1K8, 17J21, 22K8, and 26K20. Fluorescence in situ hybridization (FISH) to normal human lymphocyte chromosomes was used to map the genomic PAC clones to chromosome 19p13.3. Fine mapping was performed using radiation hybrid screening by PCR (Research Genetics, Inc.). Primer sequences (5′-GCTGCAGGAGCTGAAGATG; 5′-GTGGCTGGAGTTGTCCCTTA) were designed to yield a 120 bp product spanning the first exon/intron junction. Interphase FISH analysis was performed on paraffin sections of 20 sporadic ovarian and breast tumors as well as 10 normal ovary and breast specimens.

Antibody Development: A recombinant GST-fusion protein to the N-terminal region of APC2 (aa 1- 249) was produced in *E. coli* using the pGEX-4T-2 vector, isolated, and released by protease cleavage (Pharmacia Biotech). This protein was used to inoculate both rabbit and chicken (Rockland Inc., Gilbertsville, PA). Both the rabbit serum and IgY collected from the chicken eggs was affinity purified on an antigen coupled CnBr column (XMMR website at http://vize222.zo.utexas.edu/Marker_pages/methods_pages/affinity_col.html).

Northern Analysis: Human multiple tissue and human cancer cell line poly(A)+ RNA blots were obtained from Clontech and processed according to the supplied manufacturer's protocol using a probe to the N-terminal region of APC2.

RT-PCR: RNA was isolated by the RNAzol method (Tel-Test, Inc.). RT-PCR was performed using the Perkin-Elmer Gene Amp RNA PCR Core Kit. Primers to the N-terminal region (5′-AGGAGCTAAGGGACAACTCCA; 5′-TCCAGCAGCTCCTTGTCAAT) were designed to yield a 600 bp fragment. These primers were shown to be specific to APC2 by sequencing of the product as well as using wild-type APC as a negative control.

Western Blot: Cells were grown to confluence in 150 mm dishes, washed twice with phosphate buffered saline (PBS) and lysed for 10 minutes on ice in 1% HEPES lysis buffer containing 1% Triton-X and protease inhibitors (1 mM sodium vanadate, 50 mM sodium fluoride, and Boehringer Mannheim complete mini EDTA-free protease inhibitor cocktail tablet). Lysates were centrifuged at 14,000 rpm at 4°C. Protein content was determined by the BCA protein assay (Pierce). Western blotting was performed as previously described using either rabbit or chicken APC2 antibody at 1 μg/ml, APC Ab-1 (Oncogene) at 1 μg/ml, or β-catenin (Transduction Laboratories) at 1:1000 (24). The blots were developed using chemiluminescent detection (Pierce). Specificity of the antibodies was determined by incubating recombinant APC2 antigen (10 μg/ml) with the antibody for 1 hour at room temperature before incubating the blot.

Immunocytochemistry: SKBR3, A549, MDA-MB-157, SW480, and MDCK cells were plated on 18 mm coverslips in 12 well plates at approximately 50,000 cells/well. SKOV3 cells were transfected with 0.2 μg of APC2 using Lipofectamine Plus (GibcoBRL). In some experiments, cells were treated with 2 μM cytochalasin D (Sigma) in media for 2 hours at 37°C. Antibody blocking with the immunogen was performed as described above. Both treated and untreated cells were fixed with 2% paraformaldehyde and permeablized with 0.2% Triton. Purified chicken APC2 antibody was used at a concentration of 1 μg/ml and secondary antibody conjugated with fluorescein (Pierce) was used at 1:100. Other primary antibodies and reagents were used at the following concentrations: normal IgY (Rockland) at 1 μg/ml, monoclonal β-catenin antibody (Transduction Laboratory) at 1:100 overnight at 4°C, polyclonal anti-APC (kindly provided by P. Polakis; 3) at 1:100 overnight at 4°C, monoclonal anti-tubulin (Sigma) at 1:2000, phalloidin (Molecular Probes, Inc) at 1:200 for 15 minutes, and anti-PKCμ (Transduction Lab.) at 1:200. All primary antibodies were incubated for 1 hour and all secondary antibodies were used at a 1:100 dilution for 1 hour at room temperature unless otherwise noted above.

RESULTS

Chromosomal Localization and Fine Mapping. Using a 1 kb sequence to the N-terminal region, four PAC clones (1K8, 17J21, 22K8, and 26K20) were isolated for APC2. FISH analysis using the four clones localized APC2 to chromosome 19p13.3, which confirms the previously published chromosomal assignment (Figure 1A (7,8)). 19p13.3 is ~20 mb in size. The genomic sequence of APC2 is ~ 40 kb and the coding sequence 7 kb. APC2 was then fine mapped by radiation hybrid mapping to the 800 kb region containing markers D19S883 and WI-19632 using primers designed to span the first exon/intron junction (Figure 1B). This particular region of 19p13.3 exhibits significant loss of heterozygosity (LOH) in many different cancers and is near the Peutz-Jeghers syndrome (PJS) associated gene, LKB1/STK11. PJS is characterized by, intestinal hamartomas and increased risk of gastrointestinal, ovarian, pancreatic, and breast cancers (26). Even though there is significant LOH in this region, there are few mutations in the LKB1 gene in sporadic breast and colorectal cancers and adenoma malignum of PJS patients (21,22). In addition, although 50% of ovarian cancers contain an LOH on 19p13.3, LKB1 is not mutated indicating that another gene of significance in the development of cancer exists in this region (27). For example, marker D19S216, which is 9.5 fcM distal to marker D19S883, but not LKB1 itself, exhibits 100% LOH in sporadic adenoma malignum of the uterine cervix (23). Therefore, APC2 could be a tumor suppressor gene affected by LOH of chromosome 19p13.3.

Using PAC clone 22K8, we performed FISH analysis on the SKOV3 ovarian cancer cell line, which had reduced levels of APC2 protein, and a variety of other cancer

cell lines including those derived from breast and colon. These pilot studies showed that most had two or more copies on chromosome 19. However, although SKOV3 cells had on average 3 signals, none of them were on chromosome 19. To continue this study, we screened 20 sporadic breast and ovarian tumors by interphase FISH analysis. Remarkably, 19 of 20 specimens from ovarian cancer patients exhibited marked allelic imbalance (Table I). In contrast no significant allelic imbalance was observed in normal ovarian or breast tissue. The number of signals of the 19p telomeric marker was also significantly less than 2 in the ovarian cancer specimens. Even taking into account the bias toward slightly underestimating hybridization signals using interphase FISH, these data are consistent with published studies showing a marked loss of chromosome 19ptel in ovarian cancers and indicate an even more marked loss of APC2. Although the overall ratio of APC2/19p when averaged over 20 breast cancers was not markedly reduced (Table II), 4 of 20 breast cancer specimens did actually exhibit allelic imbalance at this locus.

Exogenous expression of APC2 in SKOV3 cells. In pilot studies we found that many cells expressed significant levels of APC2. However, the ovarian cancer cell line SKOV3 expressed low levels of APC2 as demonstrated by western blot and immunocytochemistry (Figure 2). We used SKOV3 cells to examine the distribution of exogenously expressed APC2. Upon APC2 transfection, the intensity of several bands was markedly increased as detected by western blot using a chicken antibody to APC2. Immunocytochemistry showed that APC2 transfected cells could easily be detected against the background of non-transfected cells. Exogenously expressed APC2 localized

around the nucleus and co-localized with PKCμ, a kinase known to associate with the Golgi apparatus (28) (Figure 2 A1-3). Co-localization of APC2 and PKCμ was observed for much of the Golgi stack indicating that APC2 is associated with certain regions of the Golgi where it co-localizes with PKCμ even though the Golgi apparatus is somewhat disrupted in the transfected cells. APC2 co-localized with actin filaments at the cell membrane (Figure 2 B1-3). SKOV3 cells have very few actin filaments in the cytoplasm compared to the MDA-MB-157 cells shown in Figure 5. β-catenin co-localized with exogenous APC2 in the aggregates at the Golgi apparatus whereas in untransfected cells β-catenin is localized only at the membrane (Figure 2 C1-3). This could be indicative of a transport function for APC2 as suggested in recent studies (29-31). Exogenously expressed APC2 also appears to co-localize with some perinuclear microtubules (Figure 2 D1-3).

APC2 Expression. APC2 expression was determined by RT-PCR, Northern analysis and Western analysis of both cell lines and tissue. For RT-PCR 1 ug of total RNA from each cell line or tissue was used for amplification, for Northern analysis 10 ug of total RNA and for Western analysis 60 ug of protein from each cell line. We confirmed that APC2 is expressed in a variety of tissues, including breast, colon, brain, and ovary, at both the RNA and protein level (designated as (+) Table III; 12). In some cells APC2 levels were reduced but still detectable (+/-). APC2 expression, like APC, is greatest in the brain; however, there are differing levels in different brain regions with very little expression in the cerebellum and cerebral cortex (results not shown). Lymphoid tissues

and lymphoma cell lines had no detectable APC2 at the mRNA or protein level with the exception of K-562 leukemia cells, which express low levels of APC2 (Figure 3).

Both rabbit and chicken hAPC2 antibodies were affinity purified on an antigen coupled CNBr column. Western blot analysis determined that both antibodies were specific to hAPC2 with no cross-reactivity to APC (Figure 3A). This was confirmed using the SW480 colon cancer cell line that contains a C-terminally truncated form of APC. Neither rabbit nor chicken hAPC2 antibodies detect this truncated APC protein even though it contains the conserved N-terminus. The predicted molecular weight of full-length APC is ~310 K and that of APC2 is ~245 K. In addition, APC2 is present in T84 cells, which have a homozygous deletion of the APC gene. To further determine specificity, we blocked the antibody with recombinant antigen before Western blot analysis and found that all bands are specific to APC2 (results not shown). Similarly, when preimmune IgY was used to probe Western blots no staining was observed (not shown). Western blot analysis showed that APC2 is expressed in many cells lines including SKBR3, SW480, MDCK, MDA-MB-157 and 436 (Figure 3B). HL-60 lymphoma cells have no detectable APC2 protein, which correlates with the mRNA data. A characteristic pattern of immunoreactive species was observed. Three major bands larger than 200 kD and several smaller molecular weight species of ~121, 81, and 51 kD (not shown on this blot) were present consistently. Other cell lines, for example MDA-MB-157 and SKOV3, have significantly less of all bands. The presence of multiple bands by western blotting with N-terminal APC2 antibodies is similar to that observed for APC using N-terminal APC antibodies (32). In the case of APC the multiple banding

pattern has been ascribed to a combination of multiple splice variants and to degradation products. Because several lower molecular weight species are present in cells that are transfected with full-length APC2 (Figure 2) it is likely that these bands represent degradation products. Some differences in the pattern of immunoreactive species were observed when endogenous APC2 was compared in different cell lines and when compared to exogenously expressed APC2. These variations are likely to represent variable degrees of proteolytic degradation in the different situations. However it is possible that like APC, APC2 may also have many splice variants.

The N-terminal region of APC2 contains the highly conserved dimerization domain. This region in APC has been shown to dimerize *in vitro* (33,34). To determine whether APC associates with APC2, APC2 was immunoprecipitated with the purified rabbit antibody and APC detected using APC Ab-1 in SW480 and HBL-100 cells (Figure 3C). Full-length APC was detected in HBL-100 cells and the 150 kD truncated form of APC was detected in SW480 cells. This result demonstrates that APC and APC2 can either dimerize or associate in a complex in a detergent soluble lysate.

Sub-cellular Localization of APC2. To investigate the localization of endogenous APC2 in the cell, we performed immunocytochemistry on several cell lines including SKBR3 breast cancer cells, MDCK normal canine kidney cells, SW480 colon cancer cells, and A549 lung carcinoma cells. Although both rabbit and chicken antibodies exhibited a similar staining pattern by immunocytochemistry, the chicken antibody was

exceptional and was used for these studies. Preimmune chicken IgY and antigen blocked antibody, as well as IgY prior to antigen affinity purification, were completely negative (Figure 4 A and B). Specific APC2 staining was similar in all cell lines and was observed diffusely in the cytoplasm as well as being associated with tubular structures adjacent to the nucleus that resembled the Golgi apparatus (Figure 4). Staining was also concentrated along filamentous structures and in what appeared to be lamellipodial membranes.

APC2 Association with Actin Filaments. As was observed with exogenously expressed APC2 in SKOV3 cells, endogenous APC2 co-localized with PKCμ at the Golgi apparatus (Figure 5 A1-3). A relationship between APC2 and actin filaments was observed in cells stained with phalloidin (Figure 5 B1-3). However, not all actin filaments stained and APC2 appeared to be concentrated near actin-associated membrane ruffles and lamellipodia as well as cell-cell contact sites. Treatment with cytochalasin D, an actin-disrupting agent, causes actin filaments to collapse and coalesce mostly at the cell periphery but also throughout the cell. Following treatment with cytochalasin D, APC2 remained associated with the actin filaments in MDA-MB-157 cells (Figure 5 C1-3).

Endogenous APC is localized at the tips of microtubules in MDCK cells and is not associated with actin filaments (1). However, overexpression of APC results in the decoration of microtubules throughout the cell (16,35). Consistent with this, cytochalasin D treatment does not affect APC staining but disruption of microtubules with nocodazole does (1).

DISCUSSION

Subcellular localization of hAPC2

hAPC2 is diffusely distributed in the cytoplasm, is localized to the Golgi apparatus, and is associated with actin filaments. In some instances, such as lamellapodia or membrane ruffles, APC2 exhibits a punctate staining at the ends of actin filaments. Unlike APC, APC2 remains associated with the disrupted actin filaments following treatment with cytochalasin D. Recent studies show that E-APC/dAPC2 co-localizes with actin caps during Drosophila development and negatively regulates wingless signaling in the epidermis (20,38,39). These data suggest that even though sequence similarity is low, hAPC2 and dAPC2/E-APC may be functional homologues and that both may be involved in actin-associated events such as motility as well as in β-catenin signaling.

In contrast, endogenous APC localizes near the ends of microtubules in a punctate pattern but does not co-localize with actin (1); however, when over-expressed, APC associates with microtubules throughout the cell (16,35). Over-expressed APC2 forms aggregates at the Golgi apparatus and co-localizes with actin filaments as well as some microtubules. Similarly, Nakagawa *et al* (18) reported that over-expressed, epitope-tagged APCL/APC2 localizes in the perinuclear region and microtubule network. Our results indicate that as is the case with APC, APC2 localization with microtubules in the cell body may only occur when APC2 is present at very high levels. It has been suggested that APC might be involved in microtubule regulated membrane protrusion and cell migration as well as inhibition of β-catenin signaling (40). Like APC, APC2

also can inhibit β-catenin signaling (11,12). Although APC2 and APC do not co-localize at the membrane or cytoskeletal structures they are both present in the cytoplasm. Immunoprecipitation results show that they can exist in the same complex in this environment. Taken together these findings suggest an intriguing scenario in which cytoplasmic APC and APC2 regulate related microtubule and actin-based functions and β-catenin signaling either independently or in cooperation. APC and APC2 could cooperate in the cytoplasm or in association with microtubules and actin filaments respectively to control such processes as β-catenin signaling and cell motility as suggested by Barth *et al* (40). In addition interactions between microtubules and actin filaments occur during cell motility (41). The cellular location and many binding domains of APC2 suggest that it has multiple and perhaps dynamic functions. For example, recent studies located a nuclear export signal that allows APC, APC2, and E-APC to shuttle between the nucleus and the cytoplasm thus suggesting a transport mechanism (29-31).

Significance of the chromosomal location of APC2

The chromosomal localization of APC2 to chromosome 19p13.3 within 12 fcM of the telomere is significant because this region is associated with Peutz-Jeghers Syndrome (PJS) and exhibits significant loss of heterozygosity (LOH) in several sporadic cancers. Patients with PJS are more susceptible to breast, testis, gastrointestinal, and ovarian cancers (26). Loss of 19p13.3 occurs in many sporadic cancers including those of the breast and is remarkably common in sporadic ovarian carcinomas (~50%) (27). Ovarian cancers are also characterized by a high rate (~16%) of stabilizing β-catenin mutations

(42). However, mutations in the PJS gene, LKB1, are not present in most of these sporadic cancers suggesting the existence of other tumor suppressor loci in this region of chromosome 19 (21,27). Our fine-mapping analysis shows that APC2 is located in the region of markers D19S883 and WI-19632 between the LKB1 gene and the site of 100% LOH found in adenoma malignum of the uterine cervix (23). Importantly, we found significant allelic imbalance of APC2 in sporadic ovarian cancers. Therefore, like APC, APC2 could be a tumor suppressor gene important in several cancers particularly ovarian cancer.

A recent study by Townsley and Bienz (19) showed that the majority of offspring of homozygous Drosophila females carrying a weak E-APC/dAPC2 mutation die as embryos with defects similar to Wingless stimulation. Interestingly, E-APC was lost at the membrane of nurse-cells in the ovary. This loss coincided with a loss of Armadillo/β-catenin at the membrane and suggests that cell adhesion might be affected by the weak mutation in E-APC. Our data indicates that if similar mutations occur in human APC2 they may be relevant in ovarian cancer. At this time some single nucleotide polymorphisms (SNPs) have been discovered in several genes on chromosome 19p13.3 but none in the APC2 gene itself. Further studies searching for SNPs and a more formal LOH study will better determine whether APC2 is a tumor suppressor gene.

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Figure Legends

Table I: Allelic imbalance of chromosome 19p and APC2 in ovarian cancers. Interphase FISH was performed on paraffin sections from 20 sporadic ovarian tumors. The results of each specimen are shown. Each value represents the average number (from 20 cells) of FISH signals using the 19p telomeric probe and the APC2 probe. Statistical significance was demonstrated by a paired T-test with a P value of $9.82e^{-5}$.

Table II: Mean number of chromosome 19p telomere and APC2 signals in normal and tumor ovarian and breast tissues. Interphase FISH was performed on paraffin sections from 20 sporadic ovarian and breast tumors and 10 normal ovary and breast specimens. The data represents the average number of signals (from 20 cells) from 10 or 20 specimens.

Table III: Expression of APC2

APC2 is expressed in many different tissues and cell lines including brain, breast, colon, and ovary at both the mRNA and protein level as observed by northern blotting, RT-PCR, and western blotting. (+) indicates detectable APC2, (-) indicates no detectable APC2, and (+/-) indicates detectable but very low levels of APC2.

Figure 1: APC2 chromosomal localization and fine mapping

A) APC2 was mapped to chromosome 19p13.3 by FISH analysis using PAC clones identified through screening with a 1 kb cDNA fragment from the N-terminal region of APC2. B) Fine mapping using radiation hybrid screening by PCR located APC2 to the region on chromosome 19p13.3 containing markers WI-19632 and D19S883.

Figure 2: Localization of exogenously expressed APC2.

Immunocytochemical staining of SKOV3 cells transfected with APC2 shows perinuclear staining (A1) using the APC2 IgY antibody. Cells were stained for the Golgi apparatus marker PKCμ (A2). APC2 co-localizes with PKCμ at the Golgi apparatus (A3). Also note that the Golgi apparatus looks somewhat distorted in the transfected cells compared to the nontransfected cells. Double staining of APC2 (B1) and actin (B2) found APC2 localized with actin filaments in regions close to the membrane (B3; arrows). Cells were stained for APC2 (C1) and β-catenin (C2). β-catenin co-localizes with transfected APC2 in large aggregates around the nucleus (C3; arrow). In comparison, β-catenin is only found at the membrane in the untransfected cells (arrowhead). Transfected SKOV3 cells were double-stained for APC2 (D1) and tubulin (D2). Overexpressed APC2 localizes with some but not all microtubules (D3; arrows). All images are at a magnification of 630X. E) SDS-PAGE analysis was performed on a 3-8% Tris-Acetate gel. SKOV3 ovarian cancer cells express low levels of APC2 as shown by western blot with the APC2 IgY antibody. Exogenously expressed APC2 is detected a several molecular weight species.

Figure 3: Expression of APC2 and western blot of cell lysates comparing APC2 and APC.

A.) SW480 cell lysates, containing a truncated form of APC (T-APC), were used to compare affinity purified APC2 rabbit antibody (1 μg/ml) to APC antibody-1 (1 μg/ml). SDS-Page analysis was performed on a 4-12% Tris-Glycine gel. No cross reactivity could be found. The affinity purified APC2 IgY antibody gave similar results (results not shown). B.) Varying protein patterns of APC2 are observed by western blot analysis of several different cell lysates using the IgY antibody (1 μg/ml). Equal amounts of protein (60 μg) were loaded in each lane. SDS-Page analysis was performed on a 3-8% Tris-Acetate gel. Note that HL-60 and K562 lymphoma cell lines have no detectable APC2 as shown on a 4-12% Tris-Glycine gel. C.) APC2 was immunoprecipitated from SW480 and HBL-100 cell lysates using the affinity purified rabbit antibody. Rabbit IgG was used as a control. Complexes and a control lysate were analyzed for APC by western blot using APC Ab-1 (1 μg/ml). The truncated form of APC found in SW480 cells and full-length APC found in HBL-100 cells exist in a complex with APC2.

Figure 4: Immunocytochemical staining for endogenous APC2.

A) SKBR3 cells were stained using preimmune IgY (3 μg/ml). Little staining could be seen even in this overexposed image. B.) MDA-MB-157 cells stained using IgY antibody blocked with APC2 protein. C.) MDA-MB-157 cells stained for APC2 using APC2 IgY antibody (1ug/ml). APC2 can be seen concentrated along filamentous structures as well as concentrated along the membrane (arrows). D.) A549 cells were stained with APC2 affinity purified IgY antibody (1 μg/ml). The arrow indicates staining resembling the

Golgi apparatus surrounding the nucleus. E.) A549 cells stained for APC2 as above. The box indicates a region of small vesicles/particles concentrated in a lamellipodial membrane. F.) A549 cells again stained for APC2. The arrows indicate staining resembling the Golgi apparatus surrounding the nucleus and staining along actin filaments.

Figure 5: APC2 localization at the Golgi apparatus and actin filaments.

A549 cells double stained with APC2 and PKCμ or phalloidin. Cells were treated with 2 μM cytochalasin D for 2 hr. A1.) PKCμ clearly stains the Golgi apparatus. A2.) APC2 localizes to the Golgi apparatus. A3.) Double staining shows APC2 co-localization with PKCμ at the Golgi apparatus. B1.) Phalloidin staining of actin filaments in MDA-MB-157 cells. B2.) APC2 is diffusely distributed in the cytoplasm and co-localizes with actin filaments. B3.) Double staining shows APC2 associated with actin filaments. C1.) MDA-MB-157 cells treated with cytochalasin D and stained with phalloidin. Actin filaments are disrupted. C2.) Cells treated with cytochalasin D and stained for APC2. APC2 is disrupted. C3.) Double staining shows that APC2 remains associated with actin filaments following treatment with cytochalasin D.

Table I: Allelic imbalance of chromosome 19p and APC2 in ovarian tumors

Specimen	Chromosome 19p telomere	APC2
1	2.05	0.95
2	1.6	0.55
3	1.45	0.7
4	1.0	0.7
5	1.3	0.7
6	1.65	0.45
7	1.2	0.4
8	1.0	0.1
9	0.8	0.15
10	0.15	1.75
11	0.4	0.25
12	0.85	0.2
13	1.2	0.55
14	0.9	0.05
15	0.85	0.2
16	1.5	0.4
17	1.45	0.3
18	1.25	0.4
19	1.0	0.2
20	0.75	0.25
Mean	**1.12**	**0.46**

T value 4.9056
P value 9.8187e-5

Table II: Mean number of chromosome 19p telomere and APC2 signals in normal and tumor ovarian and breast tissues

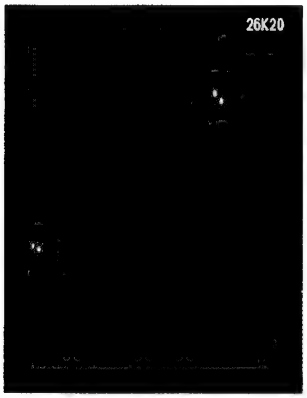
Tissue	Chromosome 19ptel	APC2	Ratio APC2/19p
Normal ovary	1.42	1.25	0.88
Ovarian Cancer	1.12	0.46	0.41
Normal Breast	1.43	1.34	0.94
Breast cancer	1.70	1.49	0.88

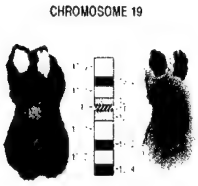
Table III: APC2 Expression

Cell Line	Type	APC2 Expression	Cell Line	Type	APC2 Expression	Tissue	APC2 Expression
A1N4	Breast	$+^{2}$	ZR-75-1	Breast	$+^{1}$	Colon	$+^{2}$
BT20	Breast	$+^{1}$	SW480	Colon	$+^{1,2}$	Ovary	$+^{2}$
HBL-100	Breast	$+^{1,2}$	LS-123	Colon	$+^{1}$	Testis	$+^{2}$
MCF-7	Breast	$+^{2}$	T84	Colon	$+^{1}$	Spleen	$-^{2}$
MCF-10A	Breast	$+^{2}$	A549	Lung	$+^{1,2}$	Periph. Bl. Leuk.	$-^{2}$
MDA-MB-134	Breast	$+^{2}$	Calu-3	Lung	$+^{1}$	Brain	$+^{2}$
MDA-MB-157	Breast	$+/-^{1}$	G361	Melanoma	$+^{2}$	Sm. Intestine	$+^{2}$
MDA-MB-453	Breast	$+^{1,2}$	SKOV3	Ovary	$+/-^{1}$	Prostate	$+^{2}$
MDA-MB-231	Breast	$+^{2}$	U87	Brain	$+^{1}$	Thymus	$-^{2}$
MDA-MB-436	Breast	$+^{1}$	A-431	Epidermoid	$+^{2}$		
MDA-MB-468	Breast	$+^{1,2}$	MOLT-4	Leukemia	$-^{2}$		
MDA-MB-435	Breast	$+^{1}$	Burkitt's Raji	Lymphoma	$-^{2}$		
SKBR3	Breast	$+^{1,2}$	HL-60	Leukemia	$-^{1}$		
T47D	Breast	$+^{1,2}$	K-562	Leukemia	$+/-^{1}$		

1 = protein, 2 = RNA
+/- = low levels

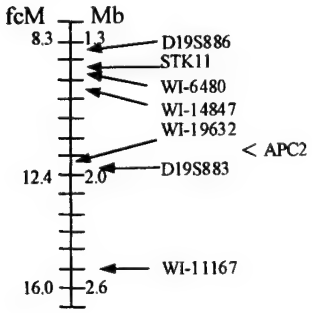
A)

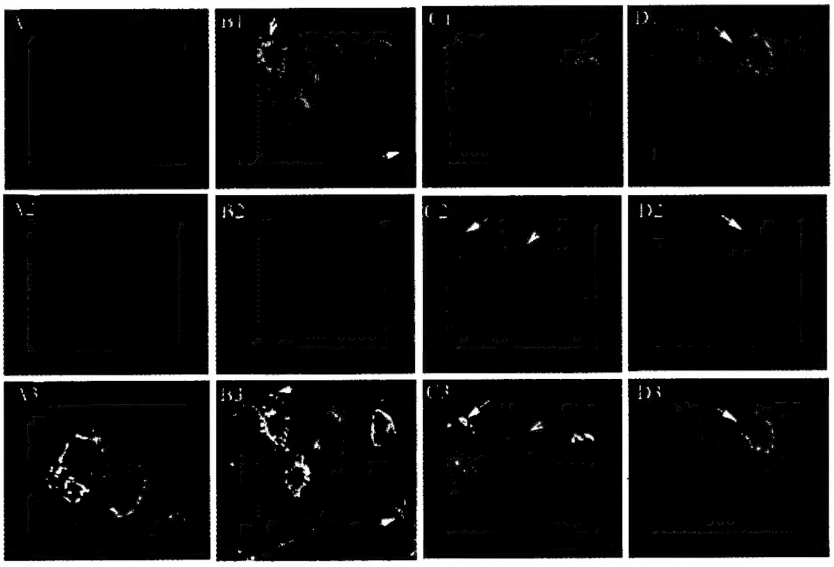




Chromosome 19p13.3

B)

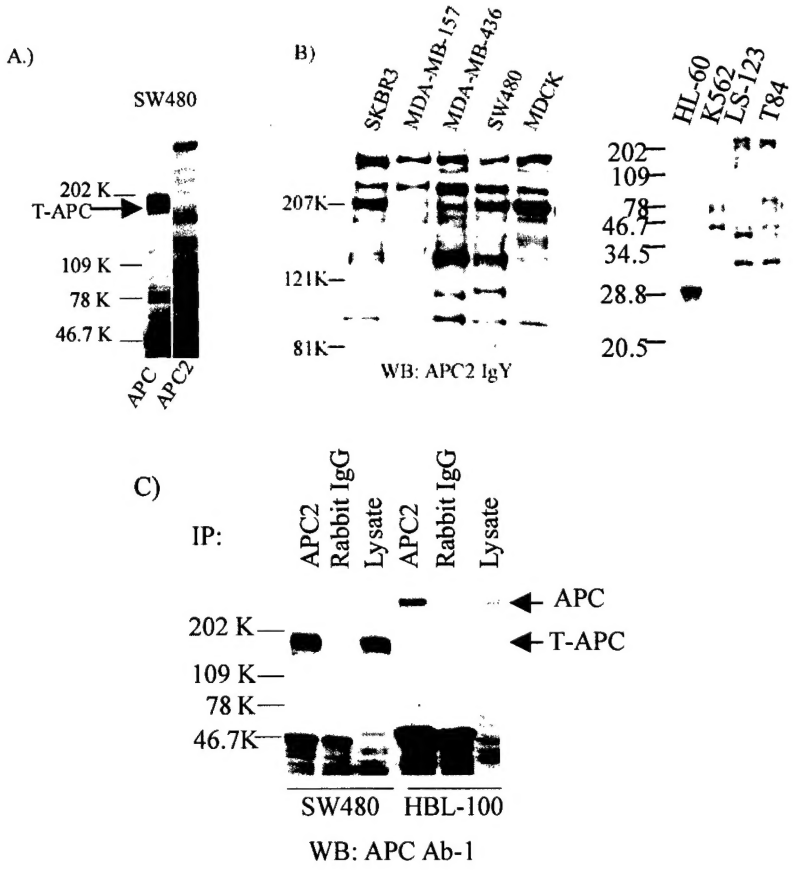




E

SKOV3 Cells

WB: APC2


A.)
SW480
202 K
T-APC
109 K
78 K
46.7 K
APC
APC2
B)
SKBR3
MDA-MB-157
MDA-MB-436
SW480
MDCK
HL-60
K562
LS-123
T84
207K
121K
81K
202
109
78
46.7
34.5
28.8
20.5
WB: APC2 IgY
C)
IP:
APC2
Rabbit IgG
Lysate
APC2
Rabbit IgG
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APC
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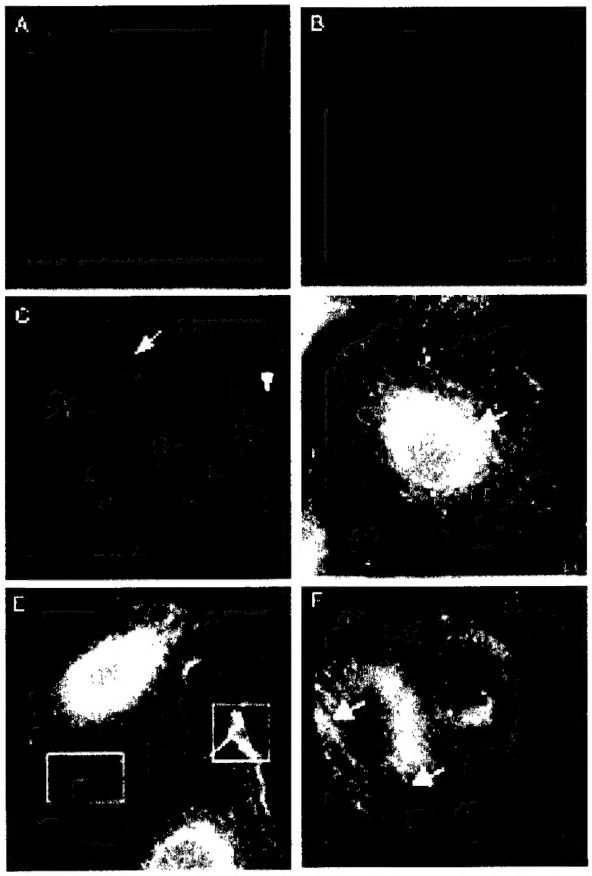
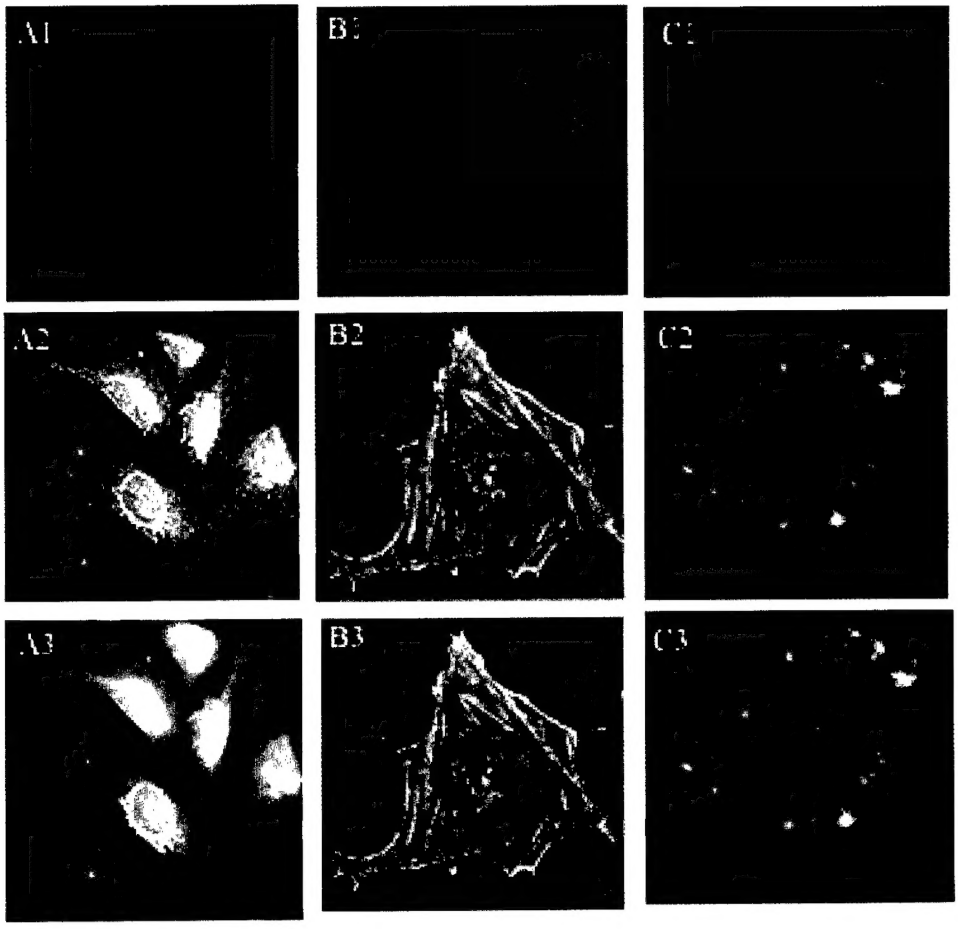
202 K
109 K
78 K
46.7K
SW480
HBL-100
WB: APC Ab-1


A
B
C
E
F


A1
B1
C1
A2
B2
C2
A3
B3
C3